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First isolation of *Brucella pinnipedialis* and detection of *Brucella* antibodies from bearded seals (*Erignathus barbatus*)

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ABSTRACT: *Brucella* species infecting marine mammals was first reported in 1994 and in the years since has been documented in various species of pinnipeds and cetaceans. While these reports have included species that inhabit Arctic waters, the few available studies on bearded seals (*Erignathus barbatus*) have failed to detect *Brucella* infection to date. We report the first isolation of *Brucella pinnipedialis* from a bearded seal. The isolate was recovered from the mesenteric lymph node of a bearded seal that stranded in Scotland and typed as ST24, a sequence type associated typically with pinnipeds. Furthermore, serological studies of free-ranging bearded seals in their native waters detected antibodies to *Brucella* in seals from Chukchi Sea (1990-2011; 19 %) and Svalbard (1995-2007; 8 %), whereas no antibodies were detected in bearded seals from the Bering Sea, Bering Strait or from captive bearded seals.

KEY WORDS: Antibodies · bearded seal · *Brucella pinnipedialis* · isolation · MLST

INTRODUCTION

The isolation of *Brucella* from marine mammals was first reported in 1994 from four free-ranging harbour seals (*Phoca vitulina*), two harbour porpoises (*Phocoena phocoena*) and a common dolphin (*Delphinus delphis*), all inhabiting Scottish coastal waters (Ross et al., 1994) and from an aborted foetus born to a captive bottlenose dolphin (*Tursiops truncatus*) in the USA (Ewalt et al., 1994). Since these initial reports, *Brucella* infection has become recognised in cetaceans and pinnipeds inhabiting many of the world's oceans (Foster et al., 2002; Nymo et al., 2011) and two species, *Brucella ceti* and *Brucella pinnipedialis*, have been described for isolates with cetaceans and seals as preferred hosts, respectively (Foster et al.,

2007). These species are genetically distinct from *Brucella* associated with terrestrial mammals (Whatmore et al., 2016).

With respect to Scottish coastal waters, *B. pinnipedialis* has been recovered from the other resident species, grey seals (*Halichoerus grypus*) as well as from hooded seals (*Cystophora cristata*), which are occasional visitors to the region (Foster et al., 1996; 2002). The isolation of *B. pinnipedialis* has also been reported from hooded seals in their native Arctic waters and from harbour and grey seals elsewhere in Europe (Nymo et al., 2011). Further afield, *B. pinnipedialis* has been cultured from other pinniped species including Pacific harbour seal (*Phoca vitulina richardsi*) (Garner et al., 1997), ringed seal (*Pusa hispida*), harp seal (*Pagophilus groenlandica*) (Forbes et al., 2000) and California sea lion (*Zalophus californianus*) (Goldstein et al., 2009). Serological studies provide further presumptive evidence that *Brucella* infections are widespread amongst other pinniped species, including some resident in the Southern Hemisphere (Nymo et al., 2011). Taken together, culture and serological evidence (Foster et al., 2002; Nymo et al., 2011), indicate that *Brucella* is endemic in many of the marine mammals that inhabit the world's open oceans and seas. Seropositive animals, however, can be due to immunological cross-reactions, in particular *Yersinia enterocolitica* serotype O9, however, this strain has not been recovered from marine mammals to date (Ross et al., 1996 and pers. obs. GF) to an organism from a different genus; thus the isolation of *Brucella* by cultural methods, remains the gold standard of definitive proof of infection in different hosts and discrete populations of marine mammals.

There have been few reports on studies of *Brucella* infection in bearded seals (*Erignathus barbatus*) to date, but where performed, no evidence of exposure was

found (Calle et al., 2008; Tryland et al., 1999). Bearded seals are members of the Phocidae family and represent the only species within the genus *Erignathus*. They have a patchy circumpolar distribution throughout the Arctic and subArctic between 45 and 85° N. Two sub-species are recognised, *Erignathus barbatus barbatus*, which ranges from the central Canadian Arctic eastwards to the central Eurasian Arctic and *Erignathus barbatus nauticus*, which ranges from the central Canadian Arctic westwards to the Laptev Sea, Russia. The availability of sea ice to breed, moult and rest on, in shallow water areas, is thought to be an important factor governing the distribution of this benthic-feeding seal (Burns, 1981; Kovacs, 2016). In a review of their extralimital records, bearded seals have been reported from the Netherlands, France and Spain in the Eastern Atlantic and the island of Rügen in the Baltic Sea (van Bree, 2000). Sightings in the UK are rare, with most modern reports occurring around the Scottish coast, including the Shetland and Orkney Islands and single sightings from the Isle of Mull, Aberdeenshire and Fife (JNCC/Defra, 2013).

This paper documents the first recovery and characterisation of *B. pinnipedialis* from a bearded seal. The results of a serological study of free-living bearded seals in Arctic waters and captive members of the species kept at the aquarium ‘Polaria’ in Tromsø, Norway are also presented.

MATERIALS AND METHODS

Bearded seal necropsy

In early February, 2012, a stranded bearded seal (M61/12) was reported to the Scottish Marine Animal Strandings Scheme (SMASS). The juvenile male animal had

stranded dead at Annachie Lagoon, St Fergus on the Aberdeenshire coast of the north-eastern Scottish mainland (57°34′10.74″N 001°49′22.02″W) and represented the first report of a stranded bearded seal in Scotland since records began in 1992. The carcase was transported to SAC Consulting Veterinary Services, Inverness for a post mortem examination performed according to a standard protocol (Dierauf, 1994). Samples of brain, lung, liver, spleen, kidney, mesenteric lymph node, urinary bladder and small intestine were cultured on Columbia sheep blood agar (CSBA) (Oxoid, Basingstoke, UK) and Farrell's medium (FM) (Farrell, 1974), incubated at 37 °C in air with 5 % added CO_2 as described previously (Foster et al., 2002). Plates were examined for growth, daily, for 4 days and at frequent intervals thereafter up to 14 days. Isolates with colonial appearance typical of *Brucella* were tested initially for Gram reaction, cellular morphology, acid-fastness with the modified Ziehl-Neelsen stain, agglutination with *Brucella abortus* antiserum (Remel, Basingstoke, UK) and ability to grow in air without added CO_2. Further testing included urea hydrolysis, H_2S production, inhibition by basic fuchsin at 1/50,000 and 1/100,000, agglutination with monospecific antisera A and M and lysis by phages TB, Wb, BK2, Fi, Iz and R/C all at Routine Testing Dose. Multilocus sequence typing (MLST) using a 9 locus scheme was performed as described previously (Whatmore et al., 2007). Tissue samples for histological examination (whole brain, trigeminal ganglion, skin, thyroid gland, adrenal gland, urinary bladder, spleen, lung, kidney, heart, and pancreas) were collected, trimmed and processed routinely through graded alcohols and embedded in paraffin wax prior to sectioning (5µm), mounting on glass microscope slides and staining with haematoxylin and eosin. Blood was collected from the left ventricle of the heart using a sterile needle into heparinised and plain vacutainers 6

ml vacutainers (BD, Wokingham, UK) for serology and urine analysis was performed using the Combur 9 Test (Roche, Burgess Hill, UK).

Serology

The Alaska Department of Fish and Game Ice Seal program recovered serum from heart blood samples from subsistence harvested bearded seals. Seals were shot on sea ice by Alaska native hunters, as allowed under the Marine Mammal Protection Act of 1972, in the Chukchi and Bering Strait off the north and northwest coasts of Alaska, during May, June, July and October. In addition, 17 seals were sampled immediately post mortem during 1978-1979 scientific collections conducted April-June by the Outer Continental Shelf Environmental Assessment Program during United States National Oceanographic and Atmospheric Administration cruises in the Bering Sea under National Marine Fisheries permit #194 (Figure 1). Samples from bearded seals from Svalbard were obtained from both dead and live animals. Sixteen animals were shot on the ice as part of Norwegian scientific catches to address stocks and diets for different seal species and their role in the marine ecosystems and interactions with fisheries (1992-1995). Blood was collected on the ice when cutting the main blood vessels to the fore flippers during standard bleeding-out procedures of seals (Tryland et al., 1999). From live bearded seals (pups; 1995-2007) blood was obtained from the extradural intra-vertebral vein using an 80 mm needle (14 gauge, 2.1 mm) mounted on a 50 ml syringe. Blood was transferred into blood collecting tubes (Venoject, Terumo, Leuven, Belgium) and serum was prepared (3000 g, 15 min) and stored at -20 °C until analysis. Sex and age category (pup < 1 year, juvenile < 3 years, adult > 3 years) were known for some or all of the seals at each location (Table 1). Furthermore, blood samples were obtained from 5

bearded seals kept in captivity at the aquarium “Polaria” in Tromsø. Blood was drawn from the plantar venous plexus of the hind flippers, using a 0.8 x 50 mm needle and blood collecting tubes (Venoject). Serum was prepared by centrifugation at 3 000 g for 15 min and serum stored at -20 °C until analysis.

These animals, initially captured in the wild in Svalbard, had been kept in captivity since they were approximately 5 weeks of age; the seals interact extensively with humans through training and feeding (Stokke, 2010). They were 9-10 years of age at the time of sampling and had been trained to tolerate handling and blood sampling (Table 1).

Serum samples (n = 205) were analyzed for anti-*Brucella* antibodies with a Protein A/G indirect enzyme-linked immunosorbent assay (ELISA) as described previously (Nymo et al., 2013a). The mean optical density (OD) of duplicate wells was expressed as a percentage of the reactivity of a seal positive control: ([OD sample/OD positive control] × 100) = percent positivity (%P). The cut-off was 73.6 %P.

Statistical Analysis

All statistical analysis was performed in JMP 11 Pro (SAS Institute, Medmenham Marlow, UK).

RESULTS

Bearded seal necropsy

The carcase of M61/12 was fresh and had been chilled, but not frozen, prior to necropsy, two days after notification. The animal was 149 cm in total length, 79 cm girth behind the front flippers and in moderate to poor body condition with a mid-sternal blubber thickness of 16 mm.

Hair loss ranged from complete over the ventral surface of the animal through partial coverage over the flank with bilateral symmetrical zones of alopecia, back to almost complete hairloss over the perilumbar region. The head and neck exhibited almost full coverage, excepting significant alopecia periocularly and over dorsal muzzle. In addition, the foreflippers exhibited partial alopecia over the carpal and phylangeal regions. No regions showed evidence for hair regrowth.

The oesophagus and stomach contained a notable amount of sand, and marine debris comprising a fragment of worn black plastic sheet 45 mm long and a single round pebble 1 cm in diameter was recovered from the stomach. No prey items were found. Thyroid glands were grossly unremarkable. The lungs and cerebral vessels were markedly congested, the bladder mucosa was grossly reddened and the urine was turbid and dark red in colour and a high level of haemoglobin (ca 50 erythrocytes per µL) was detected with the Combur 9 Test. The brain showed diffuse dilation of cerebral vessels but the cerebrospinal fluid was unremarkable.

Bacteriology

Small numbers of colonies typical of *Brucella* were recovered from the mesenteric lymph node on CSBA and FM after four days. In addition, *Vibrio alginolyticus* was recovered from multiple tissues. Cells of suspect *Brucella* colonies were tiny Gram negative cocco-bacilli, which were acid-fast when tested in the modified Ziehl-Neelsen stain. Agglutination was obtained in slide tests with *B. abortus* antiserum.

The strain required CO_2 for growth, was urease positive, H_2S negative and A dominant. Growth was inhibited by basic fuchsin at 1/50,000 and 1/100,000 and cultures were completely lysed by Tb phage, partially lysed by Wb, BK2 and Iz, with no lytic effect with Fi and R/C. The strain was identified by MLST as *Brucella pinnipedialis* sequence type (ST) 24.

Histopathology

The most significant histological change in M61/12 consisted of moderate, multifocal granulomatous and eosinophilic meningo-encephalitis within the brain, often centred on degenerate or intact nematode parasite larvae, with perivascular cuffing and multifocal haemorrhages. The nematode larvae were not identified but gross morphology of worms seen in the stomach were consistent with Anasakid nematodes, *Pseudoterranova bulbosa* or *Contracaecum osculatum*. It is plausible that the granulomatous foci in the brain were the result of aberrant tissue migration of L4 larval stages from these species. Mild, multifocal histiocytic and eosinophilic pneumonia (likely parasitic) was also noted along with moderate splenic histiocytosis with mild lymphodepletion. The skin lesions consisted of mild epidermal hyperplasia with follicular atrophy with no evidence of vasculitis or dermal necrosis. Moderate to marked thyroid follicular hyperplasia and moderate to marked bilateral adrenocortical hyperplasia were present. The most significant lesions and likely cause of death, were multiple granulomatous foci in many regions of the brain consistent with migrating nematode larvae. Overall, the seal appeared to have indications of chronic morbidity and malnutrition/pica which, given the extralimital nature of this case, could be due to pathogen exposure and/or inadequate feeding capacity.

Serology

Antibodies to *Brucella* were detected in 22 of 200 (11 %) serum samples collected from wild bearded seals in Alaska and Svalbard (Table 1). Sixteen of the seropositive seals came from 86 (19 %) animals that were subsistence harvested in the Chukchi Sea between 1990 and 2011; one juvenile female, two juvenile males, four adult females, one adult male, five females with unknown age class, one male of unknown age and two animals of unknown sex and age (Table 1). The other seropositive bearded seals, 6 of 76 (8 %), were all captured in the Svalbard archipelago during the period 1995 to 2007. The positive animals were three female and two male pups and the mother of one of the female seropositive pups. It is not known whether the mothers of the other seropositive pups were amongst the animals sampled. Antibodies to *Brucella* were not detected from any of the 38 bearded seals subsistence harvested in the Bering Strait or collected in the Bering Sea or from the five animals kept in captivity at “Polaria” (Table 1). *Brucella* antibodies were detected in the blood collected from the necropsied animal (M61/12).

The average %P of the seropositive seals was 87,7 %P (SD 9,4) and the average %P of the seronegative seals was 25,9 %P (SD 21,6). The material included six mother/pup pairs from Svalbard sampled between 06.05.95 and 25.05.95. One mother/pup pair was seropositive (mother: 93,2 %P, pup: 88,8 %P). The remaining pairs had %P values below the cut-off, however, the %P of the pup could be predicted from the %P of the mother with the following formula: pup = -75,8 + 1,7*Mother, r^2 = 0,84.

DISCUSSION

This study documents for the first time the recovery of *Brucella* from a bearded seal, as well as the first serological evidence of *Brucella* exposure in this host. Antibodies were detected in sera from two of the four groups of free-ranging bearded seals sampled; the Chukchi Sea (19 %) and the Svalbard archipelago (8 %), however, they were not detected from 38 bearded seals from the Bering Strait region or the Bering Sea (Table 1). A previous small-scale study also failed to detect *Brucella* antibodies from six bearded seals taken during a subsistence hunt at St Lawrence Island in the Bering Sea (Calle et al., 2008), so evidence of exposure to *Brucella* in this region remains lacking (Figure 1). The Pacific bearded seals are not distinct populations, they move from the Bering Sea through the Bering Strait with the advancing and retreating ice edges. The detection of seropositive bearded seals from the Chukchi Sea therefore may be significant for *Erignathus barbatus nauticus* across their entire area. Another serological study for *Brucella* in bearded seals did not detect antibodies from two locations in the North Atlantic, while antibodies were detected in the other three sympatric species sampled; hooded, harp and ringed seals (Tryland et al., 1999).

Typing of the *Brucella* isolate by MLST (Whatmore et al., 2007) demonstrated that it belonged to the ST24 lineage of *B. pinnipedialis*. Sequence type 24 is the less common of two STs isolated predominantly from pinnipeds (Groussaud et al., 2007) and has previously been found associated with harbour seals, grey seals and a minke whale (*Balaenoptera acutorostrata*) which stranded in Scotland and from harbour seals and a beluga whale (*Delphinapterus leucas*) from North America (Groussaud et al., 2007; Whatmore et al., 2017).

Brucella-associated pathology was not found either grossly or histologically, although histology was not performed on the lymph node and an association of *B. pinnipedialis* with the death of this animal was not established. This is in line with previous findings, which have revealed a paucity of pathologies following *Brucella* isolation from pinnipeds, including several apparently healthy harbour seals which had been shot by fishermen (Foster et al., 2002). In contrast, a broad range of pathologies have been reported for *Brucella* infection of various cetacean species which include lymphocytic meningoencephalitis, sub-cutaneous lesions, blubber abscessation, liver abscess, hepatic and splenic necrosis, macrophage infiltration in liver and spleen, lymph node inflammation, pneumonia, peritonitis, mastitis, osteomyelitis, spinal discospondylitis, diseased atlanto-occipital joint, endocarditis, epididymitis and abortion (Foster et al., 2002; Nymo et al., 2011; Guzman-Verri et al., 2012).

In vitro work has revealed differences between the classical terrestrial *Brucella* strains and *B. pinnipedialis*. The *B. pinnipedialis* reference strain NCTC 12890 and *B. pinnipedialis* hooded seal strains were eliminated from murine and human macrophage cell lines, and a human epithelial cell line within 72-96 h (Larsen et al., 2013b). Even more rapid elimination patterns were observed in hooded seal primary alveolar macrophages (Larsen et al., 2013a) and epithelial cells (Larsen et al., 2016). *Brucella pinnipedialis* NCTC 12890 was also found to be attenuated in the BALB/c *Brucella* mouse model (Nymo et al., 2016). The reduced virulence in these models, when compared to the terrestrial virulent strain *Brucella suis* 1330 (Larsen et al., 2013b; Nymo et al., 2016), is in line with the limited virulence of the *B. pinnipedialis* strains in their natural hosts (Foster et al., 2002). Five seropositive pups were detected in the present study, all from Svalbard. The sampling took place in May and peak birthing for

bearded seals at Svalbard is in early May. The pups are thereafter weaned in approximately 24 days (Kovacs, 2016). The seropositive pups were hence of very young age. At least one of the seropositive pups in the present study was the pup of a seropositive mother and a strong relationship was identified between the titres (i.e. antibody levels) in the mothers and the pups in the six mother/pup pairs from Svalbard. These findings suggest a transfer of maternal antibodies between mother and pup. Seals have an endotheliochorial placenta (Stewart and Stewart, 2009) where solely 5-10 % of the maternal antibodies are transferred to the fetus *in utero*. Passive immunity via the colostrum is therefore essential in species in which the type of placentation impedes contact between maternal and foetal circulation systems, hindering the transfer of antibodies (Tizard, 2000). Indeed, when evaluating total IgG levels in harbour seal mother and pups the mothers showed a decreasing trend during lactation, while the total IgG levels in the pups were low at birth and higher at the end of lactation (Ross et al., 1993). In dogs, which also have an endotheliochorial placenta (Stewart and Stewart, 2009), pups from *Brucella canis*-infected bitches have antibodies against *B. canis* (Carmichael and Kenney, 1970). In animals having an epitheliochorial placenta the young are born virtually agammaglobulinemic, however, after receiving colostrum calves from dams with high and low *Brucella*-antibody levels had corresponding high and low *Brucella*-antibody levels (Sutherland et al., 1990). Maternal transfer of antibodies against phocine distemper virus has been shown in harbour seals (Garnier et al., 2014) and maternal and pup antibody titres were shown to be strongly correlated in Scottish grey seals (Pomeroy et al., 2005). Even though the number of mother/pup pairs investigated in the present study is low, our findings and the literature supports that the *Brucella*-antibody levels of the pups are likely a reflection of the *Brucella*-antibody levels of the mothers and the result of maternal transfer of antibodies rather than a vertical infection of the pups. However, further bacteriology work on organ samples from mother and pup pairs is needed in order to draw any conclusions on this matter. For hooded seals, however, no relation was found between *Brucella* serostatus and ovulation rate or neonatal body condition (Nymo et al., 2013b).

Bearded seals are largely solitary animals (Kovacs, 2016). Ringed seals and hooded seals, from which *B. pinnipedialis* has been isolated and anti-*Brucella* antibodies detected (Forbes et al., 2000; Nymo et al., 2013b) are also generally described as being largely solitary (Kovacs, 2002; Miyazaki, 2002), though all three of these species do gather in the same areas where habitat is suitable for breeding, moulting and foraging. Contrary to cetaceans where vertical transmission of *Brucella* was suggested (Ohishi et al., 2016), no evidence of vertical transmission of *Brucella* in true seals has been reported. Furthermore, the solitary behaviour of bearded seals suggests that opportunities for *Brucella* transmission between conspecifics are restricted. Altogether, this re-enforces the possibility that *Brucella* infection may be acquired from the environment, possibly via diet, as suggested previously (Lambourn et al., 2013; Nymo et al., 2013b). In contrast, harp seals have also been shown to harbour infections with *B. pinnipedialis* (Forbes et al., 2000; Tryland et al., 1999) but this species demonstrates a much stronger tendency to congregate (Lavigne, 2002) and transmission between conspecifics cannot be excluded.

Brucellosis is a significant zoonotic infection, which causes a broad range of manifestations, especially associated with farmed animals and their products, infected with *Brucella melitensis, B. abortus* and *B. suis*, but also *Brucella canis* contracted from dogs. Whilst, there have been three reports of human infections with marine mammal *Brucella*, none have involved *B. pinnipedialis*. Human infection has been reported in a laboratory infection scenario with ST23, a clade predominantly associated with porpoises, while naturally occurring infections have been reported only with ST27 (Whatmore et al., 2008), only isolated thus far from bottlenose

dolphins (*Tursiops truncatus*) and California sea lions in the USA (Whatmore et al., 2017) and recently, from a single bottlenose dolphin in the Mediterranean (Cvetnik et al., 2016).

While the lack of human infections with *B. pinnipedialis* are in contrast to the findings with *B. ceti* and the classical *Brucella* spp. mentioned above, the zoonotic potential of *B. pinnipedialis* remains unknown at present. It is advisable, therefore, that those working with bearded seals and other pinniped species consider the infectious nature of the genus and follow appropriate safety procedures (Dierauf and Gulland, 2001).

In conclusion, we report the first isolation of *Brucella pinnipedialis* from a stranded extra-limital juvenile male bearded seal. In contrast to cetaceans, reports of *Brucella*-associated pathology in pinnipeds is lacking. Our study also provides novel serological evidence for *Brucella spp.* exposure in free-ranging bearded seal subpopulations. Future serological surveys and the isolation and characterization of *Brucella* isolates from stranded and free-ranging bearded seals, as well as other ice seals (ringed seal; ribbon seal; spotted seal) are needed to better understand the significance of *Brucella* infection in these northern pinnipeds

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